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# Purification and characterization of thermostable organic solvent-stable protease from *Aeromonas veronii* PG01

K. Divakar, J. Deepa Arul Priya, Pennathur Gautam *

*Centre for Biotechnology, Anna University, Chennai 600 025, India*

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ABSTRACT

A mesophilic bacterium, *Aeromonas veronii* PG01, isolated from industrial wastes produced an extracellular thermostable organic solvent tolerant protease. The optimum condition for cell growth and protease production was pH 7.0 and 30 °C. The protease produced was purified 53-fold to homogeneity with overall yield of 32%, through ammonium sulphate precipitation, ion-exchange and gel permeation chromatography (GPC). The molecular weight, as determined by GPC–HPLC, was found to be about 67 kDa. SDS-PAGE revealed that the enzyme consisted of two subunits, with molecular weight of 33 kDa. The protease was active in broad range of pH from 6.0 to 10.0 with optimum activity at pH 7.5. The optimum temperature for this protease was 60 °C. The enzyme remained active after incubation at 50–60 °C for 1 h. This enzyme was stable and active after incubation with benzene and it was activated 1.3- and 1.5-fold by n-hexane and n-dodecane, respectively. This protease was inhibited completely by the classic metalloprotease inhibitor 1,10-phenanthroline and partially by the metal chelator EDTA but not by the serine protease inhibitor PMSF. The PG01 protease was found to contain 1.901 mol of zinc per mole of enzyme upon analysis by Inductively coupled plasma-optical emission spectroscopy. The thermostable and solvent tolerance property make it an attractive and promising biocatalyst for enzyme mediated synthesis.

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## 1. Introduction

Currently, enzymes have attracted the world attention due to their wide range of industrial applications. Approximately 80% of all industrial enzymes are hydrolytic in nature and are used for depolymerization of natural substances i.e., the breaking down of complex molecules into simpler ones. Proteases constitute one of the most important groups of hydrolytic enzymes, accounting for about 60% of the total enzyme market sectors especially in detergent, food, leather, textile, dairy and pharmaceutical industries [1,2].

Microbial proteases have drawn the attention of many researchers due to their tremendous application potential in various industries [3]. *Aeromonas* spp. is commonly found in the water and food samples. The isolation of *Aeromonas* from raw milk [4] and food products [5] has been reported earlier. Most strains of *Aeromonas hydrophila* produce single heat-stable metalloprotease [6] and some strains also secrete an unrelated serine protease [7]. There are few reports describing the purification and characterization of zinc metalloprotease from *Aeromonas* sp., [8,9]. Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity [10]. Most metalloproteases are zinc containing proteins, which are involved in virtually all aspects of metabolism of the different species of phyla [11].

Most of the industrial processes are carried out at higher temperature, where many enzymes are often unstable above certain physiological conditions [12]. There is ample scope for screening for thermostable proteases with organic solvent stability, which can resist harsh industrial process conditions. The advantage of conducting biotechnological processes at elevated temperature is that it reduces the risk of contamination by common mesophiles. It has also a significant influence on the bioavailability and solubility of organic compounds [13]. Under normal aqueous conditions, protease catalyses the hydrolysis of peptide bond, but the reaction proceeds in the reverse direction (i.e., synthesis of peptide bond) in water restricted media [14]. Reports on hydrolytic enzymes that are naturally stable in the presence of organic solvents have emerged recently [15–17].

In the present study, a mesophilic bacterium which produces an extracellular thermostable protease was isolated from milk industry wastes and identified as *Aeromonas veronii* by 16S rDNA sequencing. Various culture conditions for protease production were investigated. The purification, biochemical characteristics,

* Corresponding author. Tel.: +91 44 27452270; fax: +91 44 27453903.
*E-mail addresses:* pgautam@annauniv.edu, ksvinod29@yahoo.com (P. Gautam).

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thermostability and organic solvent stability of the purified protease were studied.

## 2. Experimental

### 2.1. Materials

Enzyme substrates and inhibitors were procured from Sigma (St. Louis, USA) and chemicals for media preparation were purchased from Hi-Media (Mumbai, India). Ion-exchange chromatography sorbent DEAE Ceramic HyperD was purchased from PALL Life Sciences (St. Louis, USA). HPLC grade solvents and other reagents were from SRL (Mumbai, India) and Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

### 2.2. Methods

#### 2.2.1. Isolation and screening of organism producing thermostable protease

The organism used in this study was isolated from industrial wastes, collected from a milk processing unit (stagnated milk, leaks in hot pipelines), in Chennai, India. The sample (100 μl) was suspended in 1 ml of sterilized water. The suspended sample was spread on nutrient agar plate containing (g/l): peptone 2.0, NaCl 5.0, yeast extract 2.0, beef extract 1.0, and bacteriological agar 15.0. The bacterial colonies were purified and screened for their protease production on skim-milk agar plate containing (g/l): peptone, 2.0; NaCl, 5.0; yeast extract, 2.0; beef extract, 1.0; skimmed milk powder, 10.0; and bacteriological agar 15.0. The plates were incubated at 37 °C for 48 h. Colonies that had formed a clear zone around their margins were picked up as protease producers. Nine isolates showed a clear zone in skim-milk agar plate. The positive isolates were further screened for production of extracellular thermostable protease by assaying the protease activity in liquid culture using azocasein as substrate at 60 °C. The strain PG01, which showed maximum activity, was selected for further study. The stock culture of the strain was maintained on glycerol stocks (50%, v/v) and stored at −20 °C.

#### 2.2.2. Identification of microorganism

The taxonomic characteristics of this strain were examined according to Bergey's Manual of Determinative Bacteriology [18] and 16S rDNA gene sequencing [19]. A homology search was performed with Genbank database using BLAST algorithm. Alignment of 16S rDNA sequences of PG01 with other *Aeromonas* sp., strains was carried out using CLUSTALW program at the European Bioinformatics Institute server [20]. The neighbour-joining phylogenetic tree was constructed with PHYLIP program [21]. The isolate was identified as *A. veronii* PG01. The 1546 bp 16S rDNA gene sequence of *A. veronii* PG01 has been submitted to Genbank database, with the accession number GQ334329.

#### 2.2.3. Optimization of culture conditions

Optimal conditions for growth and protease production were studied using nutrient broth media containing (g/l): peptone 2.0, NaCl 5.0, yeast extract 2.0, and beef extract 1.0, at temperature ranges of 25–40 °C, pH ranges of 6.0–10.0, with increments of one unit, inoculum density of 0.5–5% (v/v) (O.D 0.5), with increments of 0.5% (v/v). The flasks were incubated on refrigerated rotary shaker at 140 rpm for 24 h. The samples were withdrawn at 2 h interval. The growth was monitored by measuring the optical density at 600 nm and cell free supernatant was analysed for protease activity.

#### 2.2.4. Enzyme production

For the production of protease, a 250 ml Erlenmeyer flask containing 50 ml of nutrient broth (pH 7.0) was inoculated with 2% (v/v) of stationary-phase culture (O.D 0.5) prepared in same medium and incubated in an orbital shaker at 140 rpm for 12 h at 30 °C. The culture supernatant was collected after centrifugation at 10,551 × *g* for 15 min at 4 °C and used for further purification.

#### 2.2.5. Enzyme and protein assay

Protease activity was measured according to the method described by Sarath et al. [22] with some modifications. Briefly, 250 μl of a suitable dilution of the enzyme solution was added to 250 μl of 5 mg/ml azocasein (Sigma) in 50 mM sodium phosphate buffer (pH 7.5). The mixture was incubated at 60 °C for 30 min, and the reaction was terminated by adding 1.2 ml of 10% (w/v) trichloroacetic acid (TCA). To allow precipitation of undigested substrate the mixture was placed on ice for 10 min. The precipitate was removed by centrifugation at 12,000 × *g* for 5 min. After adding 500 μl of supernatant to equal volume of 1 M NaOH, the absorbance at 440 nm was recorded with a HITACHI U-3210 spectrophotometer. A blank was prepared by the same procedure, TCA being added at zero time. One unit of protease activity was defined as the amount of enzyme required to produce an increase in absorbance of 0.1 at 440 nm. The protein content was determined by Bradford's method using the Bio-Rad assay reagent, with bovine serum albumin (BSA) as standard [23].

#### 2.2.6. Purification of protease

Proteins were precipitated from culture supernatant by the addition of solid ammonium sulphate upto 30% saturation. The resultant mixture was centrifuged at 12,557 × *g* for 15 min at 4 °C, and the supernatant was brought to 60% saturation. The precipitated protein was collected by centrifugation at 12,557 × *g* for 15 min at 4 °C. It was then dissolved in minimum volume of buffer A containing 10 mM sodium phosphate buffer (pH 7.5) and dialyzed against the same buffer. The dialyzed protein sample was applied to DEAE Ceramic HyperD, which had been equilibrated with 20 column volume of buffer B (20 mM sodium phosphate buffer, pH 7.5). The protein applied to the column was washed subsequently with buffer B to remove any unbound proteins and eluted with a step wise concentration gradient of NaCl (25, 50, 75, 100, 125, 150 and 200 mM) in the buffer B. Fractions (3.0 ml) were collected and aliquots were assayed for protease activity. Protease active fractions were pooled, dialyzed against the buffer B. Dialyzed protein sample (in buffer B) was freeze dried (Lyophilizer – Martin Christ Alpha 1-2 LD plus) at a temperature of −53 °C at a pressure of 0.09 mbar for 6 h and stored at −20 °C. The purified sample from ion-exchange column was further purified by gel permeation chromatography. The chromatographic separation was carried out in Agilent 1100 HPLC system equipped with quaternary pump, automatic injector, thermostatized column compartment and photodiode array detector. A Zorbax GF-250 (4.6 mm × 250 mm i.d., 5 μm particle size, Agilent technologies) gel filtration chromatography column was used. The column was previously equilibrated with 0.2 M sodium phosphate buffer (pH 7.5). The protein was eluted with same buffer at flow rate of 0.5 ml/min at 25 °C. Eluted proteins were monitored using diode array detector at 280 nm and protein peaks found were collected in a thermostatized fraction collector.

#### 2.2.7. RP-HPLC analysis of purified protease

Purity of the enzyme was confirmed by RP-HPLC analysis using Agilent 1100 HPLC system. The purified protease was applied on to C-18 column (Zorbax C-18, 4.6 mm × 250 mm i.d., 5 μm particle size, Agilent technologies). For elution, buffer C consisted of 0.1% (v/v) trifluroacetic acid (TFA) in water and buffer D consisted of 0.1% (v/v) TFA in acetonitrile. After loading, the column was washed with 2% (v/v) buffer D for 2 min, to elute any unbound protein. The bound proteins were eluted from column using a 40 min linear gradient from 2 to 100% (v/v) buffer D at the constant flow rate of 1 ml/min.

Column temperature was maintained at 25 °C and column eluent was monitored at 280 nm.

*2.2.8. Polyacrylamide gel electrophoresis and activity staining of purified protease*

The purified protease was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the molecular weight of the protein. SDS-PAGE was carried out according to method of Laemmli [24] with some modifications. A discontinuous gel containing 4 and 10% (w/v) of acrylamide in stacking (pH 6.8) and resolving gel (pH 8.8) were used respectively. For reducing SDS-PAGE, samples were boiled for 5 min with sample loading dye containing disulphide bond reducing agent (β-mercaptoethanol) before electrophoresis. For non-reducing SDS-PAGE, the reducing agent was omitted and the samples were not boiled before loading. Relative molecular weight of the enzyme was estimated by comparison with protein molecular weight markers (LMW kit, Amersham Pharmacia Biotech). After electrophoresis, the gel lane loaded in duplicate under non-reducing was used for activity staining and other part of the gel was stained with Coomassie brilliant blue R-250 (CBB-R250) to locate protein bands. For activity staining, the gel was first equilibrated in 25 mM sodium phosphate buffer (pH 7.5), for 30 min and then the gel was immersed in equilibration buffer containing 1% (w/v) gelatin for 1 h at 37 °C, in order to allow the substrate to diffuse into the gel. The gel was then transferred to a clean petri dish and incubated at 37 °C for 1 h for the digestion of the gelatin by the active protease. After incubation the gel was stained with CBB-R-250. Protein band with protease activity in the gel was visualized as clear zone of hydrolysis [25].

*2.2.9. Molecular weight determination by gel filtration chromatography*

In addition to SDS-PAGE, the molecular weight of purified protease in native form was determined by HPLC–GPC analysis using above mentioned HPLC system. Purified protease was injected into Zorbax GF-250 (4.6 mm × 250 mm) gel filtration chromatography column (Agilent technologies). The column was previously equilibriated with buffer E (0.2 M sodium phosphate buffer (pH 7.5) containing 0.2 M NaCl). The protein was eluted with the same buffer at a flow rate of 0.5 ml/min. Eluted proteins were monitored using diode array detector at 280 nm. Molecular weight of the purified protein was calculated by comparing the retention time of protease with retention of molecular weight markers (phosphorylase b 97 kDa, albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 30 kDa, α-lactalbumin 14.4 kDa).

*2.2.10. Biochemical properties of PG01 protease*

*2.2.10.1. Effect of pH and temperature on activity and stability.* The effect of pH on the purified protease activity was determined by incubating the purified enzyme between pH 6.0 and 12.0 under the standard assay conditions. The buffers used were sodium phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 7.5–9.0), glycine–NaOH buffer (pH 9.0–11.0) and phosphate–NaOH buffer (pH 11.0–12.0). For pH stability studies, purified enzyme was pre-incubated in 50 mM appropriate buffers for 4 h at 30 °C. The residual activity was measured at standard assay conditions. Activity of enzyme incubated in sodium phosphate buffer (pH 7.5) was assumed as 100%.

The effect of temperature on enzyme activity was examined at different temperatures (20–80 °C) for 30 min at pH 7.5. The effect of temperature on stability of the enzyme was determined by incubating the enzyme samples at 30, 40, 50 and 60 °C for 8 h. Aliquots were withdrawn at 1 h time interval to test the residual activity at standard assay conditions. The relative activity of the enzyme was determined by comparing the activity of enzyme before incubation at different temperatures.

*2.2.10.2. Effect of organic solvents on PG01 protease.* One milliliter of purified enzyme was incubated in the absence and presence of 1.0 ml of organic solvents at 30 °C with constant shaking at 180 rpm for 5 h. Aliquots were taken from these mixtures and the residual activities were determined at standard assay conditions. Relative activity was calculated by comparing the activity of enzyme incubated under same conditions, without organic solvent.

*2.2.10.3. Effect of metal ions.* To determine the effect of metal ions on protease activity, various metal salts ($HgCl_2$, $ZnCl_2$, $CuSO_4$, $CaCl_2$, $MnSO_4$, $MgSO_4$, NaCl, KCl, and $FeCl_2$), were added to purified protease at final concentration ranging from 1 to 10 mM and pre-incubated at 30 °C for 30 min. The residual activity was measured at standard assay conditions.

*2.2.10.4. Effect of inhibitors and reducing agents on activity of PG01 protease.* The effects of inhibitors and disulphide bond reducing agents were determined by incubating the purified enzyme in presence of protease inhibitors and reducing agents at 1–10 mM concentration at 30 °C for 30 min. Additives used were: –SH bond reducing agents (dithiothreitol (DTT), β-mercaptoethanol), inhibitors of metalloproteases (1,10-phenanthroline (OP) and ethylenediaminetetraacetic acid (EDTA)), serine proteases (phenylmethylsulfonyl fluoride (PMSF)) and cysteine proteases (iodoacetamide (IAA)). The residual activity was measured at standard assay conditions. Activity of the enzyme determined without additives, under similar conditions was assumed as 100%.

*2.2.10.5. Effect of surfactants and oxidizing agents on activity of PG01 protease.* The effects of non-ionic (Tween-20, Tween-80 and Triton X-100) anionic surfactants (SDS) and oxidizing agent (hydrogen peroxide ($H_2O_2$)) on protease were determined by pre-incubating the enzyme at varying concentrations of surfactants (0.1, 0.25 and 0.5% (w/v)) and oxidizing agent (0.5, 1, 1.5 and 2% (v/v)) at 30 °C for 1 h. The residual activity was measured at standard assay conditions.

*2.2.10.6. Substrate specificity.* The substrate specificity of the PG01 protease was investigated by using casein, BSA (bovine serum albumin), gelatin, egg lysozyme, keratin as substrates. Protease activity was assayed by mixing 0.05 ml of the purified enzyme with 0.4 ml of dd$H_2O$ and 0.5 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 2 mg/ml of each substrate. After incubating at 60 °C for 30 min, the reactions were stopped by adding 0.5 ml of trichloroacetic acid (TCA) (10%, w/v) and allowed to stand at room temperature. After 15 min, centrifugation was done for 15 min at 12,000 × *g*. The absorbance of the supernatant was read at 280 nm. The relative hydrolytic activity toward casein was taken as control [26].

*2.2.10.7. Determination of autolytic activity.* To determine autolytic activity of PG01 protease, the purified enzyme was pre-incubated at 30 and 60 °C for 4 h. The residual activity measured under these conditions and RP-HPLC analysis (RP-HPLC method used was same as described above) of pre-incubated samples were compared to determine autolytic activity.

*2.2.10.8. Determination of zinc content.* Zinc determination was performed using inductively coupled plasma spectroscopy (ICP-OES). The purified protein was acid hydrolysed by heating at 50 °C in 20% (v/v) nitric acid for 18 h. The concentration of zinc in the buffer in which the samples were purified was subtracted from the concentration of zinc in purified enzyme [27]. The glassware used for

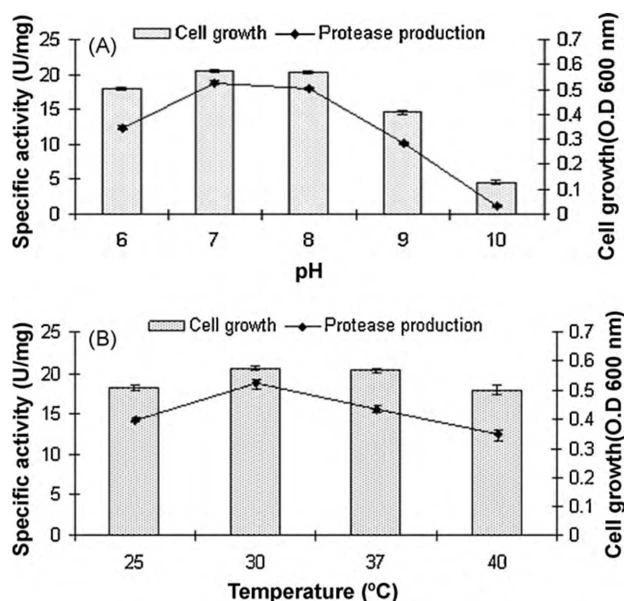


**Fig. 1.** (A) Effect of pH on cell growth and protease production and (B) effect of temperature on cell growth and production of protease.

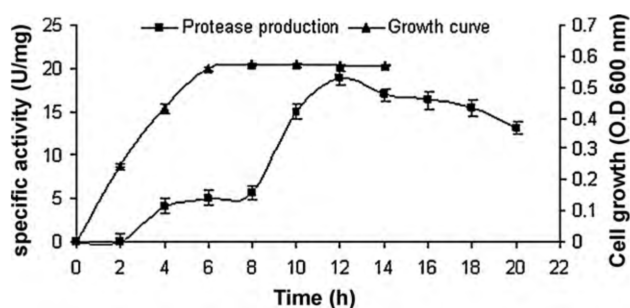


**Fig. 2.** Time course of protease production and growth of *A. veronii* PG01 under optimized culture conditions.

this study was boiled in 5% (v/v) HCl for 3 h to remove adventitious metal ions.

*2.2.11. Statistical analysis*

All the experiments were carried out in triplicate and standard deviation for each experimental result was calculated using Excel spreadsheets (Microsoft office 2003 package). The error bars in the figures represent standard deviation ($n = 3$).

## 3. Results and discussion

### 3.1. Isolation and identification of thermostable protease secreting microorganism

The strain PG01 used in this study was isolated from milk industry waste. Samples collected were plated onto skim-milk agar plates. Colonies showing clear halos were further screened for production of thermostable protease by azocasein assay at 60 °C. Morphological and biochemical characteristics showed, it as an aerobic, gram negative, motile organism. In accordance with Bergey's Manual of Determinative Bacteriology and 16S rDNA sequence analysis, the strain was identified as *A. veronii*. Comparison of the 16S rDNA sequence among available stains of *Aeromonas* species showed a high homology to *A. veronii* CHS207. A phylogenetic tree was constructed with 16S rDNA sequence of strain PG01 and other *Aeromonas* species using neighbour-joining.

### 3.2. Optimization of culture conditions

Extracellular protease was detected over a broad pH range (pH 6.0–10.0), with the optimum production of protease and bacterial growth exhibited at pH 7.0–8.0 (Fig. 1A). The optimum pH for the production of protease determined in this study is in agreement with optimum pH for the protease from *A. hydrophila* [28]. Nascimento and Martins [29] reported an optimum pH of 8.0 for protease production by the thermophilic *Bacillus* sp. strain SMIA-2.

The optimum temperature for the production of protease and bacterial growth was investigated from 25 to 40 °C. Maximal cell growth and protease production were found to be 30 °C (Fig. 1B). The incubation at 25, 37 and 40 °C was found to decrease the production of protease. The optimum temperature for the protease produced by *Bacillus* sp. MIG was found to be 30 °C [30], whereas for *A. hydrophila* it was found to be 20 °C [28]. The temperature was found to influence the secretion of the extracellular enzymes. This may be due to changing the physical properties of the cell membrane [31].

An inoculum size of 2% (v/v) yielded maximum protease activity. Further increase in inoculum size decreased protease production. Protease secretion started during early stationary phase (6–8 h). Repression of enzyme synthesis during the early stages of growth, followed by derepression in late exponential or early stationary phase, is a common feature of extracellular enzyme formation in bacteria [32]. Under optimal conditions, maximum protease production reached after 12 h incubation and prolongation of fermentation was accompanied with gradual decrease in enzyme activity (Fig. 2). A progressive decrease in proteolytic activity with increasing fermentation time could possibly be due to cessation of production, as enzymes are primary metabolites, and it could also be due to enzyme deactivation [33].

### 3.3. Purification of protease

Purification of protease was achieved by ammonium sulphate precipitation (30–60% saturation), anion exchange chromatography and gel permeation chromatography. In this three steps purification, the enzyme was purified to 53-fold with 32% recovery and specific activity of 1004 U/mg protein. The results of purification are summarized in Table 1. In ion-exchange chromatography, 125 mM NaCl fraction gave maximum proteolytic activity. No lyoprotectant was used for lyophilisation and 90% of initial protease activity was recovered after lyophilisation step. Purity of the enzyme was confirmed by RP-HPLC analysis. It was eluted as a single peak with retention time of 29.48 min in a reverse-phase C-18 column as shown in Fig. 3. A zinc metalloprotease from *Aeromonas caviae*, purified by ammonium sulphate precipi-

**Table 1**
Purification table for *A. veronii* PG01 protease.

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Culture supernatant | 5 | 94 | 18.8 | 100 | 1 |
| Ammonium sulphate | 0.56 | 63.20 | 112.8 | 67.2 | 6 |
| Ion-exchange chromatography | 0.045 | 32.50 | 772.2 | 34.6 | 41 |
| Gel permeation chromatography | 0.03 | 30.12 | 1004 | 32 | 53.4 |

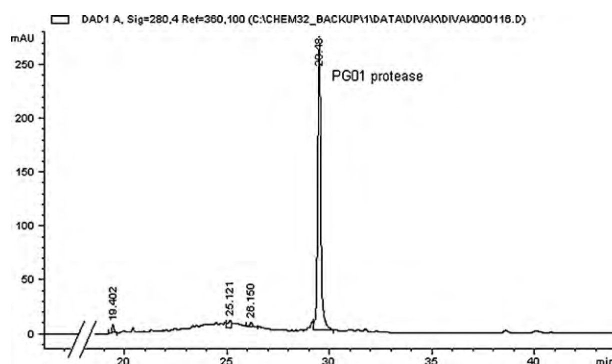


**Fig. 3.** RP-HPLC analysis of purified PG01 protease.

tation and two subsequent gel filtration chromatography methods was reported earlier by Toma et al. [9]. Soo-Jin et al. [7] purified a serine protease from *A. hydrophila* Ni39 by acetone precipitation, ion-exchange chromatography and gel filtration chromatography. The fold purification of Ni39 serine protease was 18.7 with 19.9% recovery. The fold purification and yield of PG01 protease were comparatively higher than Ni39 protease. There are also other reports of *Aeromonas* proteases purified by ammonium sulphate precipitation and ion-exchange chromatography [34,35].

### 3.4. Polyacrylamide gel electrophoresis and molecular weight determination

The purified protease was checked for presence of any subunits by comparing the molecular weight obtained under non-reducing and reducing conditions. The electrophoretic mobility of protein under reducing condition corresponds to the monomeric molecular weight of 33 kDa (Fig. 4A). Under non-reducing conditions, the protein migrates as a dimer with molecular mass of 66 kDa and protease activity was confirmed by zymography (Fig. 4B). The molecular weight of the native enzyme determined by GPC–HPLC analysis was found to be 67 kDa (data not shown); this was in agreement with native molecular mass calculated from non-reducing SDS-PAGE. This suggests that the purified enzyme may be a dimeric protein, similar to those described for proteases of *A. hydrophila* ATCC 7966, NRC 505 and Ni 39 [6,7]. However, the native molecular weight of the enzyme was different from those strains. The molecular mass and subunit composition of the extracellular protease vary between the *Aeromonas* species. The zinc and temperature-stable

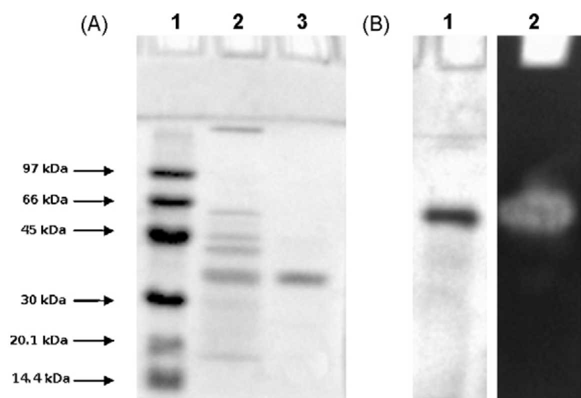


**Fig. 4.** Polyacrylamide gel electrophoresis analysis. (A) Reducing SDS-PAGE, lane 1, protein molecular mass markers: phophorylase b (97.0 kDa), albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa); lane 2, 30–60% $(NH_4)_2SO_4$ precipitated proteins; lane 3, purified PG01 protease. (B) Non-reducing SDS-PAGE; lane 1, Coomassie stained purified protease; lane 2, activity stained PG01 protease.

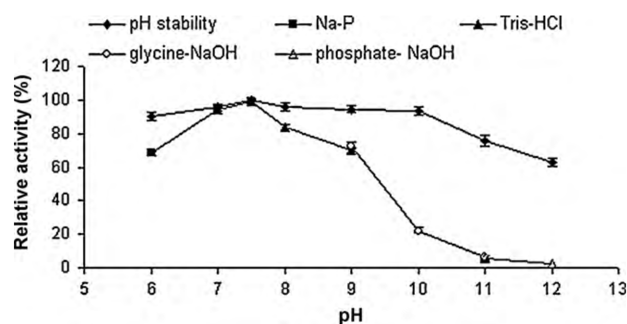


**Fig. 5.** Effect of pH on activity and stability of PG01 protease in appropriate pH buffers.

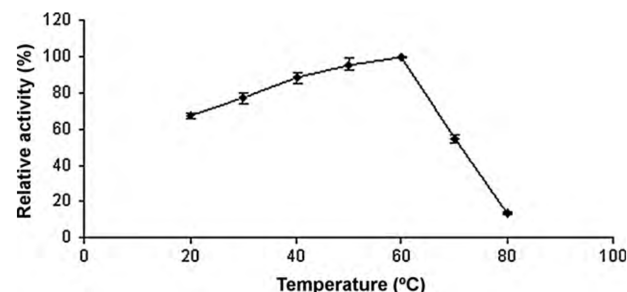


**Fig. 6.** Effect of temperature on activity of PG01 protease.

proteases of *A. hydrophila* were monomeric proteins with molecular mass of 19 and 38 kDa [8,36]. The molecular weight of protease from *Aeromonas salmonicida* and *A. sorbia* were reported as 87.5 and 67.7 kDa, respectively [35,37].

### 3.5. Biochemical properties of PG01 protease

#### 3.5.1. Effect of pH on activity and stability of PG01 protease

The purified protease had pH optima at 7.5 and the enzyme presented only 20% of its maximum activity at pH 10 (Fig. 5). But pH had very little effect on the stability of this enzyme. Not much loss of activity was observed when the enzyme was incubated from pH 6 to 10 at 30 °C for 3 h (Fig. 5). Optimal pH of PG01 protease was similar to those produced by *A. caviae* and *A. sobria* [9,38]. But in case of *A. hydrophila* Ni39 protease, it exhibited maximum activity at pH 9.0 [7]. Many proteases are reported to exhibit stability in broad range of pH [39,40].

The effect of temperature on protease activity was measured at temperatures ranging from 20 to 80 °C. Optimum activity of the purified enzyme was observed at 60 °C, while only about 53% of maximum activity was observed at 70 °C (Fig. 6). It retained 85 and 65% of its activity after incubation for 5 h at 40 and 50 °C, respectively (Fig. 7). The optimum temperature of this protease was similar to that of protease from *A. hydrophila* Ni39 [7], *A. caviae* [9]

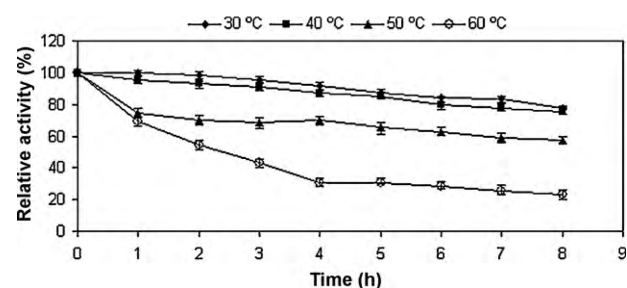


**Fig. 7.** Effect of temperature on stability of PG01 protease.

**Table 2**
Effect of organic solvents on activity of PG01 protease.

| Organic solvents | log *P* | Residual activity (%) | |
|---|---|---|---|
| | | Incubation for 2 h | Incubation for 5 h |
| Control | – | 100 | 100 |
| DMSO | −1.30 | 650.2 ± 1.26 | 41.1 ± 0.98 |
| Methanol | −0.76 | 280.5 ± 0.95 | 19.5 ± 0.92 |
| Acetonitrile | −0.33 | 38.4 ± 2.30 | 29.6 ± 1.56 |
| Ethanol | −0.24 | 34.7 ± 0.76 | 25.3 ± 0.85 |
| 2-Propanol | 0.07 | 13.2 ± 0.95 | 6.4 ± 0.52 |
| 1-Butanol | 0.80 | 10.2 ± 1.12 | 5.2 ± 0.91 |
| Benzene | 2 | 91.4 ± 1.41 | 82.7 ± 1.26 |
| Toluene | 2.50 | 112.7 ± 0.65 | 108.1 ± 1.34 |
| *n*-Hexane | 3.50 | 148 ± 0.88 | 135.5 ± 0.96 |
| *n*-Dodecane | 6.60 | 156.5 ± 0.81 | 150.2 ± 0.84 |

and was obviously higher than those of protease from *A. salmonicida* [35].

#### 3.5.2. Effect of organic solvents on PG01 protease stability

The effects of various organic solvents on the stability of PG01 protease were studied. The solvent stability of reported solvent-stable protease was carried at concentration of 25% (v/v) [17,41]. In this study, the effects of 10 organic solvents (with different log *P* values, defined as the logarithm of its partition coefficient in standard n-octane/water two phase systems [42] on the stability of protease was investigated at 50% (v/v) concentration. As indicated in Table 2, relative activity of the protease was higher in the presence of most hydrophilic and hydrophobic solvents but solvents with intermediate log *P* value decreased the enzyme activity. Therefore the stability of the protease did not follow log *P* trend. According to log *P* trend, the less hydrophobic the organic solvent, the lower activity of enzyme in the solvent [43]. This protease was more stable in DMSO and methanol (the solvents used for most synthetic reactions). Rahman et al. [17] observed that the protease from *Pseudomonas aeruginosa* strain K was quite stable in the presence of hydrophobic solvents. The solvents affect the enzyme activity by interacting with the essential water molecules surrounding the enzyme. Highly polar solvents are capable of effectively absorbing the essential water molecule from the enzyme, resulting in the loss of catalytic properties [44]. These results indicated that the PG01 protease might be a novel enzyme among reported *Aeromonas* proteases.

**Table 3**
Effect of various metal ions on PG01 protease activity.

| Metal ions | Concentration (mM) | Relative activity (%) |
|---|---|---|
| Control | None | 100 |
| $Zn^{2+}$ | 5 | 2 ± 0.23 |
| | 10 | 0 |
| $Hg^{2+}$ | 5 | 1.5 ± 0.12 |
| | 10 | 0 |
| $Mn^{2+}$ | 5 | 115.3 ± 1.25 |
| | 10 | 102.4 ± 2.01 |
| $Mg^{2+}$ | 5 | 112.7 ± 1.52 |
| | 10 | 95.0 ± 0.97 |
| $Cu^{2+}$ | 5 | 2.2 ± 0.08 |
| | 10 | 0 |
| $Ca^{2+}$ | 5 | 123.5 ± 1.38 |
| | 10 | 122.2 ± 1.56 |
| $Fe^{2+}$ | 5 | 56.1 ± 0.54 |
| | 10 | 50.8 ± 0.97 |
| $K^{+}$ | 5 | 101.2 ± 1.52 |
| | 10 | 100.4 ± 1.25 |
| $Na^{+}$ | 5 | 90.2 ± 0.85 |
| | 10 | 21.0 ± 0.73 |

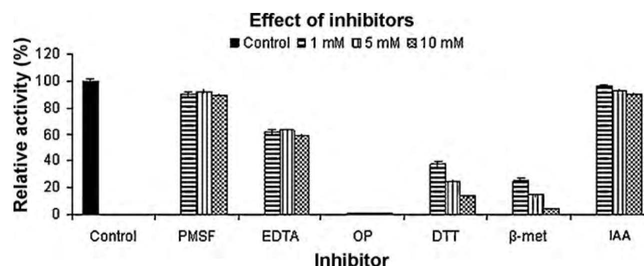


**Fig. 8.** Effect of inhibitors and reducing agents on PG01 protease. PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; OP, 1,10-phenanthroline; DTT, dithiothreitol; β-met, β-mercaptoethanol.

**Table 4**
Effect of surfactants and oxidizing agents on activity of PG01 protease.

| Reagents | Concentration % (v/v) | Residual activity (%) |
|---|---|---|
| Control | 0 | 100 |
| SDS | 0.25 (w/v) | 88.4 ± 1.25 |
| | 0.5 (w/v) | 80.3 ± 0.75 |
| Tween-20 | 0.25 | 114.1 ± 1.54 |
| | 0.5 | 112.6 ± 1.02 |
| Tween-80 | 0.25 | 117.4 ± 2.02 |
| | 0.5 | 110.2 ± 1.94 |
| Triton | 0.25 | 125.3 ± 0.84 |
| X-100 | 0.5 | 120.1 ± 1.24 |
| $H_2O_2$ | 0.5 | 100.8 ± 1.54 |
| | 1 | 94.7 ± 1.95 |
| | 1.5 | 80.2 ± 0.98 |
| | 2.0 | 51.6 ± 1.78 |

#### 3.5.3. Effect of metal ions

The effects of metal ions on protease activity were investigated. $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ increased the protease activity, whereas $Hg^{2+}$ and $Zn^{2+}$ completely inhibited the activity. It was not affected by low concentration of NaCl (5 mM) but drastically decreased at 10 mM concentration. $K^{+}$ had no remarkable effect on enzyme activity (Table 3). Increase in the activity in the presence of $Ca^{2+}$ may be due to stabilization of enzyme in its active conformation rather than it being involved in the catalytic reaction. It probably acts as a salt or ion bridge via a cluster of carboxylic groups [45]. Proteases are generally inhibited by $Hg^{2+}$ (a thiol group inhibitor). It has been reported earlier that the enzyme inhibition by $Hg^{2+}$ is not only through binding to thiol groups, but may be the result of interaction with tryptophan residue or the carboxy group of amino acid in the enzyme [46]. Several zinc peptidases are known to be inhibited by excess zinc [47,51]. The inhibition of metalloprotease by $Zn^{2+}$ has been reported earlier in *A. hydrophila* PC5 [48] and *Clostridium* sp., [49].

#### 3.5.4. Effect of inhibitors and reducing agents on PG01 protease

Enzyme inhibition studies showed inhibition of PG01 protease by OP (a zinc metalloprotease inhibitor), and EDTA (metalloprotein inhibitor), indicating that it belongs to zinc metalloprotease group of enzyme. Only 40% of its activity was reduced by EDTA, but increase in concentration of EDTA showed no further decrease in protease activity. However, the protease was insensitive to

**Table 5**
Substrate specificity of PG01 protease. BSA, bovine serum albumin; CRL, *Candida rugosa* lipase.

| Substrates | Residual activity (%) |
|---|---|
| Casein | 100 ± 0.35 |
| Gelatin | 83.3 ± 2.31 |
| BSA | 45.3 ± 1.53 |
| Lysozyme | 53.3 ± 1.13 |
| CRL | 24.3 ± 1.43 |

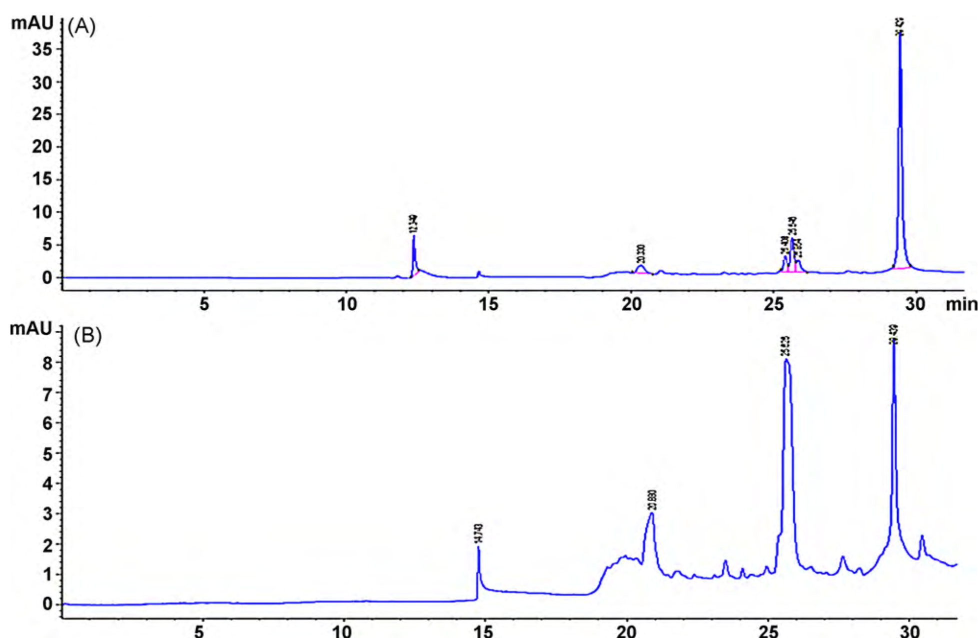


**Fig. 9.** RP-HPLC analysis to measure autolytic property of PG01 protease after incubation at 30 °C (A) and 60 °C (B) for 4 h.

the inhibitor of serine protease, PMSF and cysteine protease inhibitor, iodoacetamide. In addition to this the protease activity was decreased in the presence of –SH group reducing agents (DTT and β-mercaptoethanol). Increase in concentration of these disulphide bond reducing agents showed decrease in protease activity (Fig. 8). This suggests that the presence of disulphide bond plays significant role in protease activity. This was similar to *A. hydrophila* protease, which exhibited 49% inhibition by EDTA and 92% inhibition by DTT [36]. The effect of the disulphide bonds on the activity of *P. aeruginosa* PST-01 protease was examined [15]. Extracellular metalloendopeptidases from *Vibrio* sp., NUF-BPP1 [50], zinc metalloprotease from *H. pylori* [51] were reported to be inhibited by EDTA and 1,10-phenanthroline.

### 3.5.5. Effect of surfactants and oxidizing agent on activity of PG01 protease

The effect of detergents on the PG01 protease was studied and has been summarized in Table 4. Addition of strong anionic detergent (SDS) at 0.25 and 0.5% (w/v) exhibited a moderate inhibition of protease activity by 12 and 20%, respectively. Addition of non-ionic detergents like Tween-20, -80 and Triton X-100 showed significant increase in activity of the enzyme. Addition of $H_2O_2$ upto 1% (v/v) did not have much impact on the protease activity, but decrease in activity was observed at higher concentration. The PG01 protease showed improved stability in the presence of SDS, when compared to protease from *Bacillus licheniformis* RSP-09-37 [39] and *Vibrio fluvialis* strain VM01 [52]. VM 01 protease retained only 65% of its activity at 0.4% (w/v) SDS, whereas protease from *B. licheniformis* retained only 68% of its original activity at 0.5% (v/v) SDS.

### 3.5.6. Substrate specificity

The substrate specificity of the PG01 protease indicated that it was active on a variety of protein substrates. As summarized in Table 5 PG01 protease exhibited the highest activity towards casein. The enzyme exhibited 44 and 82% relative activities, while using gelatin and BSA as substrates, respectively. Similarly, alkaline serine proteases from *B. stearothermophilus* F1 [53] and metallokeratinase from *Bacillus subtilis* PE1 [26] were reported to exhibit their highest activity towards casein. However, the enzyme could not hydrolyse fibrous proteins such as keratin.

### 3.5.7. Determination of autolytic activity

Pre-incubation of purified protein at 30 and 60 °C for 4 h showed the loss of enzyme activity. From the RP-HPLC analysis of pre-incubated samples, it was seen the decrease in the peak area corresponding to the PG01 protease (Fig. 9). Therefore the decrease in enzyme activity could be due to autolytic property of PG01 protease.

### 3.5.8. Analysis of zinc content

Zinc content in the purified protein was determined by ICP-OES. The number of zinc atoms per enzyme molecule was estimated from protein content, assuming a molecular weight of 67 kDa. Two moles of zinc was found per mole of enzyme (1.901 g-atoms of zinc/mol i.e., 1 mol of zinc per subunit). Loewy et al. [8] and Thys and Brandelli [54] reported the presence of only one zinc atom per molecule of metalloproteases purified from *A. hydrophila* and *Microbacterium* sp., respectively. Burley et al. [55] reported the presence of two zinc ions in leucyl aminopeptidase, essential for catalysis. This protease shares few properties with well known neutral proteases like thermolysin and carboxypeptidase A. Presence of zinc ion, activity at higher temperature and neutral pH shows similarity towards thermolysin family of protease. Inhibition pattern in the presence of zinc metalloprotease inhibitor (OP) and in excess zinc, makes it comparable to carboxypeptidase A.

## 4. Conclusion

The isolated strain *A. veronii* PG01 has been found to be able to secrete extracellular protease. Although many proteases from microbial origin have been reported, to our knowledge this is the first report on purification and characterization of a thermostable organic solvent tolerant protease from *A. veronii*. This protease was purified to homogeneity by three steps purification with over all yield of 32%. Band pattern of purified protease in reducing, non-reducing SDS-PAGE suggests the presence of two identical subunits in the purified protein. Inhibition of the enzyme activity in the presence of DTT and β-mercaptoethanol proves the presence of disulphide bond in the native enzyme. Inhibition of protease by OP and zinc analysis by ICP-OES proves the PG01 protease as a zinc metalloprotease. The high stability of the PG01 protease at high

temperature, broad pH range and in presence of organic solvents proved this enzyme, an ideal candidate for use as biocatalyst in organic synthesis involving organic solvents and harsh industrial process.

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